Physiological and Behavioral Changes Associated with Arctic Ground Squirrel (AGS) Reproduction

ABSTRACT AND SPECIFIC AIMS

This project is designed to monitor the physiological and behavioral changes associated with arctic ground squirrel (AGS) reproduction. Specifically, results from this study will elucidate the timing and hormonal correlates of reproduction with the goal of establishing thresholds of reproductive hormone concentrations that could be used as markers for successful captive breeding in the future. There are four specific aims of this project: 1) genotype adult AGS currently at UAA in order to paternity test captive-bred pups; 2) monitor changes in behavior and circulating hormone levels (testosterone, estradiol, progesterone) in AGS upon emergence from hibernation; 3) pair animals for breeding immediately after emergence; and 4) monitor changes in vaginal histology during all stages of reproduction in female AGS.

The overarching goal of this project is to identify changes in behavior and physiology related to AGS reproduction in order to identify physiological markers of reproduction. Results from this investigation will be of relevance to both the basic and applied arenas of biology by increasing our knowledge of comparative reproductive biology and using that information to increase the success of captive breeding of AGS. Having a dependable supply of AGS through successful captive breeding would help to meet the need of providing research animals in the laboratory and would reduce the number of animals being caught and transported to UAA. This in turn could reduce the number of animals removed from their natural habitat as well as reduce the cost and risks associated with animal transportation.

INTRODUCTION

The arctic ground squirrel (AGS, *Urocitellus parryii*) is a model organism for numerous fields of study. Environmental studies have used AGS to investigate how animals in their natural habitat respond to changes in environmental conditions (Sheriff et al., 2010; Buck and Barnes, 2003). AGS have also been used in biomedical studies, as the mechanisms involved in hibernation have been evaluated as a potential therapy for a range of serious medical emergencies involving oxygen supply and energy demands (Drew et al., 2007). Genetic studies of AGS have identified microsatellite markers that enable AGS to be used in studies of reproduction and parentage in wild populations (Stevens et al., 1997). The purpose of my study is to track changes in physiology and behavior associated with AGS during their breeding season in order to identify specific physiological markers that can be used to establish a successful breeding colony of AGS held in captivity at UAA. It will also develop the use of microsatellite markers to create pedigrees for pups that are born from successful matings. Ultimately, this study will

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enable me to test the feasibility of captive breeding in AGS that would enable UAA and other institutions to maintain a steady and dependable supply of AGS for studies in many disciplines of science.

Considering that AGS are a major part of the arctic food chain, a better understanding of their physiology is critical. Sheriff et al. (2010) reported that females emerged from hibernation approximately 2-3 days after arousal and became pregnant within 1-4 days after emerging. This gives researchers a maximum of 7 days to catch and take blood samples from females to monitor the physiological changes associated with breeding in the spring. If physiological changes could be identified and elucidated in the laboratory, a better understanding of AGS breeding and physiology could be achieved. Not only would the ability to understand physiological changes associated with breeding help researchers understand more about these animals in their natural habitat, having the ability to breed captive animals would reduce the number of wild animals being removed from their environment.

By identifying physiological changes associated with AGS reproduction, the ability to successfully breed captive AGS would ensure a consistent and dependable supply of animals for research. As UAA expands its biomedical research enterprise, the ability to provide students and senior researchers with tools for their research is critical. A review by Drew et al. (2007) evaluated aspects of hibernation that could be used to prevent brain damage in emergency situations in which brain damage from lack of oxygen is a concern, such as cardiac arrest. By providing a stable stock of lab animals to work with, medical students could conduct a wide range of experiments applicable to the medical field from fertility to life saving emergency room treatments. Being able to successfully breed AGS in captivity would not only provide a dependable supply of animals to work with, but it would also reduce the amount of money spent on catching and transporting AGS from the field, allowing more funding to be available for specific tools and supplies.

Part of this captive breeding effort will involve establishing the timing of female receptiveness post-emergence. To do this we will be presenting newly-emerged females with a rotation of genetically distinct males over the course of several days post-emergence, and we will perform paternity testing on any pups born from these potential matings. The paternity tests will allow us to identity the father and hence the timing of female receptiveness. In order to perform this experiment we will need to determine the genotype of potential male and female AGS housed in the UAA vivarium. Stevens et al (1997) identified eight different microsatellites markers for use in AGS, and suggested that these microsatellites could be used to track parentage in squirrels. Stevens et al (1997) also proposed the use of microsatellites in order to study reproductive success in squirrels. Our study will use these microsatellite markers for

paternity testing for pups resulting from successful matings, with the pups being the link between reproductive success and timing. This strategy could be used to construct pedigrees and study parentage in AGS offspring in the future as well.

EXPERIMENTAL DESIGN

The experimental design for this project involves five major parts: 1) identification of microsatellite markers in adult AGS currently at UAA, 2) tracking of behavioral and physiological changes in animals coming out of hibernation, 3) captive breeding of four pairs of generically distinct AGS during their breeding season, 4) monitoring vaginal histology in female AGS to identify post-ovulation receptivity, and 5) the creation of pedigree records through paternity testing for all captive-bred pups that result from successful breedings.

In order to genotype the AGS stock currently at UAA, we will prepare DNA from AGS blood samples, amplify microsatellite markers from the extracted DNA using PCR, and determine the particular alleles amplified from each squirrel by fragment analysis (performed at the DNA analysis facility at Yale U.). Approximately 1ml of blood will be drawn via cardiac puncture during the hibernation season, prior to breeding. Blood will be collected during arousal bouts, centrifuged, and white blood cells isolated for DNA extraction. DNA will be purified using commercially available extraction kits (Qiagen Blood & Cell Culture DNA Mini Kit). DNA will be amplified by PCR with primers and reaction conditions identified by Stevens et al. (1997). Methods will be similar to those used by Stevens et al. (1997) and May et al. (1997). The results will allow us to determine which males and females are genetically distinct from each other and can thus be used in this experiment.

If there are not eight genetically distinct AGS – four males and four females – at UAA, then we will employ one of several options. With only two males and one female that are genetically distinct from each other, we can evaluate the timing of the greatest reproductive success. The other AGS can be bred in conditions without ambiguity, with only one male being placed with one female. In this way, we will know the pedigree of the pups that are born and the hormone levels can still be evaluated. For the three or more AGS that are genetically distinct, we can evaluate the timing of the most successful breeding by varying when the males are with a particular female.

Once distinguishable males and females are identified, they will be monitored daily for emergence from hibernation, indicated by an increase in body temperature for more than 24 hours (Sheriff et al.

2010). Once animals end hibernation, ½ ml blood samples will be collected from all animals via cardiac puncture (Buck and Barnes, 2003) twice weekly for approximately 8 weeks. At the same time that females are being sampled for blood, we will conduct vaginal lavages (Buck and Barnes, 1999) for later analysis via cytometry (details below). This sampling regime will enable collection throughout reproductive development, mating and gestation. If a female is not impregnated, this sampling regime will allow us to determine the rhythmicity of estrous. Collected samples will be centrifuged and plasma will be separated and stored at -80 C° until assayed for circulating hormone levels. Once all samples are collected, commercially available assay kits will be used to determine the concentration of the circulating reproductive hormones estradiol, progesterone and testosterone (Buck and Barnes, 2003).

AGS will be selectively paired for breeding beginning approximately two days after the female has ended hibernation and two weeks after the male has ended hibernation (Sheriff et al., 2010). If there are genetically distinct males that can be bred to a particular female, then they will be placed with the female at different times, in order to evaluate reproductive success that corresponds to different hormone levels at that time. The rotation will begin on the second day post-emergence for females: a male will be placed with the female for a period of 12 hours, followed by a 24 hour rest, and then a different male will be placed with that female for 12 hours, followed by another 24 hour rest. Rotations will continue for approximately seven days. For animals that are not genetically distinct, only one male will be placed with a female, with the same schedule: the pair will be placed together for 12 hours the first day and 12 hours the third day, with a break in between, up to seven days.

Flow cytometry of the vaginal lavage samples will be implemented to evaluate the ratio of epithelial to leukocytes cells (Buck and Barnes, 1999). Female AGS are induced ovulators, yet they undergo vaginal estrus that is characterized by an increased ratio of epithelial to leucocyte cell number. We aim to test the hypothesis that vaginal estrus is associated with increased receptivity and propensity to become impregnated. Flow cytometry is a powerful tool that we will use to evaluate the ratio of epithelial cell to leukocytes number as well as the types of white cells present; this final assessment is conducted through the use of dyes specific to different cell types.

Captive-bred pups that result from successful matings will be paternity tested, if the parents were genetically distinct, in order to correlate the hormone levels in the males and females at the time with the successful mating. Pups from successful matings between non-distinct parents can also be linked to circulating hormone concentrations during the breeding season using dates, with conception being approximately 25 days before birth (Sheriff et al., 2010).

ANTICIPATED RESULTS

From the results of this study, we expect to be able to identify a set of behavioral and physiological markers that can be used in the future to mark the time of greatest reproductive success. This is expected because of the large quantity of data that can be collected and evaluated.

It is anticipated that an adequate number of male and female AGS currently at UAA will be genetically distinct. This assumption is made based on the number of markers that we plan to analyze. We plan to genotype squirrels at eight different locations (loci) in their genomes (Stevens et al., 1997). Each of these loci are expected to reveal up to two different versions of each gene per squirrel. In order to unambiguously distinguish one squirrel from another in a small cohort, at least one of the eight loci must be specific to a given squirrel.

It is also expected that there will be changes in hormone levels in both male and female AGS and changes in vaginal histology in the female AGS reproductive tract post-emergence (Buck and Barnes, 1999, 2003). We expect to see increased concentrations of hormones that regulate reproduction, such as testosterone, progesterone and estradiol, immediately following emergence from hibernation. In non-impregnated females, we expect that the ratio of epithelial to leukocytes will vary depending on the stage of estrus. It is currently accepted that leukocyte cells decline during female receptiveness while epithelial cells increase (Laren et al., 1977; Buck and Barnes, 1999). We expect our results to show a trend in the ratio of epithelial to leukocyte cells that could be used to determine receptiveness in order to further evaluate the reproductive cycle in AGS.

It is not known whether there will be successful breedings during this experiment, as there is a narrow window of reproductive activity for AGS. The timing of reproduction in captive AGS is further complicated by the timing of emergence from hibernation, which may or may not match other AGS when they are held in captivity. However, we anticipate that the large quantity of data compiled will increase our understanding of the system such that at the very least the experiment can be modified to achieve successful breedings in the next season. For example, the conditions and timing set out in the current methods can be evaluated, and changed in order to find the correct timing of reproduction. Due to the number of animals used in this experiment, we do anticipate successful breedings, which will be used to evaluate the physiological and behavioral changes that accompanied the conception of the pups and markers can be identified. These changes will be used as markers in future captive breeding experiments to increase reproductive success of captive AGS.

BUDGET

Items	Requested	Existing Buck Grants	Totals
Microsatellite Genotyping	---	---	---
DNA extraction kit	$572	---	---
Oligonucleotide primers (Fluorescently-labeled)	$600	---	---
PCR Reagents (including Taq)	$179	---	---
PCR Reaction tubes	$100	---	---
Fragment analysis at Yale	$1/sample	---	---
Shipping to Yale	$50	---	---
Total	---	---	$1521 - $1581
Animal Care	---	---	---
Feed, Bedding, Space Rental	---	$500	---
Total	---	---	$500
Hormone Kits	---	---	---
Testosterone (x2)	---	$300 ea.	---
Progesterone (x2)	$300	$300	---
Estradiol (x2)	---	$300 ea.	---
Total	---	---	$1800
Cytometry	---	---	---
Dyes for staining	$119	$381	---
Total	---	---	$500
Total	**$2,000**	**$2,381**	**$4,381**

BUDGET JUSTIFICATION

This project is a comprehensive study of AGS reproduction, with many associated costs.

Animal care costs are a necessary item in order to properly feed and care for the animals. Quality feed and bedding will be provided to all animals in order to ensure their health and well being throughout the experiment.

The molecular and genotyping supplies are necessary in order to carry out the methods of this experiment. The goal of this project is to identify physiological markers that can be used in the future to successfully breed AGS. It is therefore necessary to have a way to identify the timing of the greatest reproductive success. This will be done by breeding genetically distinct males with a particular female at different times. We can trace female receptiveness and reproductive success to the male with the greatest number of pups through paternity testing. Although this part of the project is costly, the supplies come in quantities that will provide UAA with materials to carry out multiple experiments in the future. If this particular experiment should fail in one part, due to timing of emergence or other unexpected problems, there will be usable supplies for future experiments.

Hormone kits and stains for flow cytometry are also necessary. This project is a comprehensive study of AGS reproduction, and therefore, many aspects of biology must be examined. Reproduction is a complex process which requires evaluation of multiple factors that influence it. Therefore, these items are necessary expenses.

Any cost exceeding the allotted $2000 will be covered by existing grants provided by my mentor, Dr. Loren Buck.

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I thank Dr. Benjamin Harrison and Dr. Loren Buck with review of this grant request.

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TIMELINE

Genotyping of AGS currently at UAA..... ...January thru February

Breeding and Blood Draws..... ...Begin Mid-March

Presentation at UAA Undergraduate Research Symposium...Mid-April

Flow Cytomoetry.....Begin Mid March thru May

Paternity Testing.....Potentially Begins in April thru May

Expenditure Deadline.....May 15, 2012

Determination of Hormone Concentraions.....Mid May

Final Written Report Deadline.....May 30, 2012

Determination of Hormone Concentration